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SUBJECT OF INVESTIGATION

HETEROGENEITY OF CANCER CELL POPULATION
WITH REGARD TO THE NATURAL DRUG
RESISTANCE OF CANCER CELL

RESPONSIBLE INVESTIGATOR

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ABSTRACT

HETEROGENEITY OF CANCER CELL POPULATION WITH REGARD TO THE NATURAL DRUG RESISTANCE OF CANCER CELL

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The actual state of drug resistance of tumor cell population is a matter of "grading" when it is considered on the cellular level. Every tumor has its own peculiar grade of "natural" drug resistance and the grade is not a fixed value but it can fluctuate naturally around a certain level within a limited range of variations corresponding to the resistance of its cell population. Population analysis of a neoplastic population, after establishing single cell clones from the population, has so far demonstrated that the tumor cell population is not of a homogeneous constitution but is a heterogenous complex of cells of fluctuated grades of drug resistance. The resistance of a tumor cell population, therefore, is the averaged sum of all that of its constituents which differ from each other in their grade of resistance.

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CONTENT PAGE

I. INTRODUCTION

Discussions on the problem of drug resistance of cancer are usually concentrated on the "acquired" resistance of tumor which is the increased resistance to a certain drug arising from the application of the drug. However, during the course of comparative studies with ascites hepatomas of the rat, we encountered a remarkable fact that some of the ascites hepatomas were insensitive from the very begining to the treatment with HN_2 and its derivatives, to which others were quite sensitive. By further examinations we found that each of the tumors responded in different way to the treatment with HN_2-derivatives (1). In other words, each tumor has its own "natural" resistance to drugs and their resistance differed even among the same kind of cancer such as the hepatoma. The terminology "natural" resistance is used here to denote the resistance which is inherent in and is characteristic of each tumor from its origination.

The drug resistance of tumors, either of "natural" or "acquired" one, is recognized first by clinical or in vivo observations, and the criterion is "cure" or "regression" of tumors, but the way to approarch the actual state of the matter is in the tumor cell itself. When the resistance of tumor cells to a drug is sufficiently low, the

result of treatment with the drug will be recognized as the event of "cure" or "regression" of the tumor. When it is high enough, a dose required to induce any effects on tumor cells will surpass the tolerance dose of the host.

It has been said that the tumor is not a homogeneous but a mosaic population of cells with different characteristics. Such a "mosaic concept" of neoplastic population was supported by many biologists, mainly from their studies on the chromosome of tumors (2, 3). In fact no individual number of chromosomes of tumor cells is of absolute predominance in a tumor and, on the contrary, a wide range of variation of the chromosome number of cells exists within the neoplastic population, as shown by our previous studies supported by the Contract DA-92-557-FEC-31980. Hence, a question arises whether such a heterogeneity of neoplastic population will be extended to the natural drug resistance of tumor cells.

The present investigation aimed at the analysis of cancer cell population with regard to the natural drug resistance of its constituents, after establishing clonal tumors, as many as possible, from the cell population of tumors under question, in order to approarch the real state of natural drug resistance of tumors at cellular level.

II. MATERIALS

The present examinations were carried out, first, for the precise determination of the natural resistance of the different stock lines of the ascites hepatoma against HN_2N-oxide, an anticancerous drug, and, secondly, for the analysis of a tumor cell population regarding to the natural resistance of its constituting cells against the drug. As materials for the first experiment, 55 different strains of the ascites hepatoma of rats which were kept by serial animal passages in this laboratory were employed. They were prepared by the procedure of ascitic conversion of the azodye-induced malignant hepatomas in rats. The primary tumors of the present tumor strains originated each in separate animals and naturally all had a common normal ancestral cell, i.e. the liver cell of rats. However, their general characteristics, such as growth tempo, transplantability, ascitic picture and chromosomal constitution, differ markedly from each other (4).

The ascites hepatoma is composed of conglomerated as well as individually isolated free hepatoma cells proliferating in the accumulating ascitic fluid. The free tumor cells are conspicuously abundant in some strains of the ascites hepatoma. From these strains characterized of abundant free tumor cells, 3 tumors, i.e. AH 272, AH 414 and AH 39 (see figures 1, 2 and 3), were picked up and used as materials for the second experiment.

Recipient animals used for transplantations of the ascites hepatomas were exclusively "Donryu" rats of both sexes weighing 70-100 grams. The anti-cancerous drug tested was HN_2N-oxide exclusively.

III. METHODS

1. Examination of grade of drug resistance.

The drug resistance of ascites tumors has been determined by various means, such as volume of ascites, mitotic index or survival time of the host. However, caution should be exercised in evaluating the resistance grade without regarding other additional collateral evidences. The criterion used in the present study was the morphological change that occurred in tumor cells. As we have already reported (5) and generally accepted, HN_2 and its derivatives induce a characteristic cytological effect on tumor cells, "HN_2-effect" as designated by us. Scattering, coagulation and laceration of chromosomes, chromosome-bridge, and the formation of giant cells as well as their necrosis take the majority of the effect. These changes of tumor cells are very conspicuous, that they were used as an indicator for the examination of resistance.

The principle of our tests was that tumor cells of the ascites were brought into contact with the drug in vivo or in vitro and examined for the cytological effect caused by the drug. The dose of the drug required to induce the effect on half tumor cells present was determined —the "minimum effective dose", M.E.D.

(A) <u>In vivo test</u>. The ascites hepatoma rats were injected intraperitoneally with various doses of

HN_2N-oxide 4 days after transplantation. The tumor ascites of the injected rats was cytologically examined with Wright-Giemsa stained smears at 24 hour intervals until 96 hours after the drug injection. This procedure was repeated and the minimum amount of the drug to induce the HN_2-effect on half tumor cells, i.e. M.E.D. in vivo, was determined.

(B). <u>In vitro-in vivo test</u>. To 1.0 cc of tumor ascites tapped from 4-day-old ascites hepatoma rat, containing 100-150 million tumor cells, was added in a test tube HN_2N-oxide dissolved in 1.0 cc of physiological saline solution. After 30 minutes' incubation at 37°C., the whole content of the tube, tumor cells in 2.0 cc, was inoculated back into the peritoneal cavity of a normal rat. They began to grow in the rat whose ascites was examined under the microscope with Wright-Giemsa stained smears at 24 hour intervals until 96 hours. The minimum dose of the drug required to induce the cytological effect, i.e. M.E.D. in vitro-in vivo, was determined.

2. Establishment of clonal tumor populations.

The technique of single cell transplantation was used. The tumor ascites of stock tumor lines, containing well proliferated tumor cells like a pure culture state, was diluted with a mixture of horse serum and Hanks' balanced saline solution to such a degree that a droplet of the diluted ascites might contain only one or a few cells.

The degree of dilution varied by different intensity of population of tumor cells in the original ascites, but it was in excess of 50,000 times. Small drops of the diluted ascites was placed on a clean cover glass which was gently bridged over the moist chamber with the drops hanging. Each drop was examined under the phasecontrast microscope by magnification 80-320X for its content of cells. A droplet confirmed as including only one cell was pulled into a glass microcapillary by the aid of micromanipulator and then some of dilution medium was additionally pulled in order to make the amount of the content of the microcapillary sufficient for the usual transplantation technique. The the whole content was injected intraperitoneally into a normal recipient animal by puncturing the abdominal wall with the microcapillary. The ascitic fluid of the recipient host was examined microscopically every 4 day after the transplantation for the development of clonal tumor population. The clonal tumors thus developed and their original tumor cell population were examined for their M.E.D. and the doses were compared to each other.

3. Cold storage at -80°C. in the frozen tumor bank.

Ascites hepatoma cells were kept at -80°C. in the frozen tumor bank and the tumor cell populations that developed by transplantation of the stored cells after thawing into normal rats were examined for their M.E.D. The procedure was as follows;

To the tumor ascites of AH 39 was added pure

glycerol and mixed well. The ratio of glycerol to ascites in the mixture was 1:9 by volume. Then the mixture was sealed in a glass ampoule and directly transfered into the -80°C. deep freezer. After 48 hours' storage, the ampoule was taken from the freezer directly into the water bath at 37°C. where it was shaken for 2 minutes for the purpose of thawing. The ascites thawed was injected into the abdominal cavity of normal rats, 0.2 cc to each animal.

IV. RESULTS

1. Natural resistance of different stock strains of the ascites hepatoma against HN_2N-oxide.

The results of the examinations carried out to determine the M.E.D. of HN_2N-oxide in the various strains of the ascites hepatoma are shown in table 1. A marked variation of the drug resistance among different tumor strains was demonstrated evidently. The strains, such as AH 13, AH 66F, AH 99 and AH 130, were of the lowest M.E.D., while that of AH 149, AH 286 and AH 322 were very high. The M.E.D. in vivo of 3 strains, AH 122A, AH 122B and AH 311, could not be determined exactly since their M.E.D. in vivo were probably so high exceeding the maximum tolerance dose of the drug of the host animal. Inbetween the M.E.D. of the above-mentioned tumors, the M.E.D. of the remaining tumors were ranked, showing gradual differences as seen from the table. The table, in which the ascites hepatomas were arranged from top to bottom according to the falling order of their M.E.D. in vivo, also showed that their orders of the in vivo M.E.D. were not all in accordance with those of their M.E.D. in vitro-in vivo. However, it was suggested in this way that the refractoriness or sensitivity of tumors to a given drug was a matter of "grading" when it was considered on the cellular level and every tumor had its own peculiar resistance grade to the drug. The grade can be remarkably different

even among tumors of the same normal ancestral cell.

Table 1

M.E.D. in vivo and in vitro-in vivo as well as M.T.D. of HN2N-oxide in 55 Ascites Hepatomas

Strain	M.E.D. in vivo (mg/kg)	M.E.D. in vitro-in vivo (γ/cc)	M.T.D. (mg/kg)
AH 122A	>50.0	-	50
AH 122B	>50.0	-	50
AH 311	>50.0	-	50
AH 42B	50.0	-	50
AH 44	50.0	-	50
AH 66	50.0	10.0	50
AH 70B	50.0	-	50
AH 109A	50.0	-	50
AH 143A	50.0	-	50
AH 149	50.0	50.0	50
AH 173	50.0	5.0	50
AH 210A	50.0	-	50
AH 286	50.0	50.0	50
AH 322	50.0	50.0	50
AH 408	50.0	10.0	50
AH 423	50.0	10.0	50
AH 7974	50.0	10.0	50
AH 41A	25.0	-	50
AH 106B	25.0	-	50
AH 107B	25.0	-	50
AH 127	25.0	-	50
AH 131A	25.0	-	50
AH 131B	25.0	-	50
AH 310	25.0	10.0	50
AH 62	20.0	10.0	50
AH 62F	20.0	10.0	50
AH 21	10.0	5.0	50
AH 41B	10.0	-	50
AH 34	10.0	-	50
AH 49	10.0	10.0	50
AH 61B	10.0	-	50
AH 63	10.0	-	50
AH 136B	10.0	-	50
AH 318	10.0	10.0	50
AH 371A	10.0	-	50
AH 602	10.0	0.5	50

AH	39	7.5	1.0	50
AH	41C	5.0	-	50
AH	57B	5.0	-	50
AH	60C	5.0	-	50
AH	84A	5.0	-	50
AH	84B	5.0	-	50
AH	108A	5.0	-	50
AH	150A	5.0	-	50
AH	255A	5.0	-	50
AH	414	5.0	5.0	50
AH	7974F	5.0	-	50
AH	13	1.0	0.1	50
AH	55A	1.0	-	50
AH	65C	1.0	-	50
AH	66F	1.0	0.1	50
AH	99	1.0	0.1	50
AH	130	1.0	0.1	50
AH	272	1.0	0.5	50
AH	601	1.0	0.5	50

M.T.D. = maximum tolerance dose
-, not yet tested

2. Natural resistance of clonal populations of the ascites hepatoma against HN_2N-oxide.

In the strain AH 272, 31 rats out of 60 animals transplanted with a single tumor cell showed the development of tumor ascites and 21 clonal populations of these 31 clones were successfully submitted to the examination of their M.E.D. in vivo. In another strain AH 414, 12 clonal populations resulted from 100 single-cell transplantations and 10 clones were examined for their M.E.D. Results so far obtained from these examinations were indicated in tables 2 and 3. It was demonstrated that from the single original transplanted cell of either strain AH 272 or AH 414, 3 groups of clones were

derived which differ in their M.E.D. in vivo of HN_2N-oxide.

Table 2

Resistance to HN_2N-oxide of Clonal Sublines of the Ascites Hepatoma AH 272

No. of Clones	M.E.D. in vivo (mg/kg)
6	2.5
17	1.0
2	0.5
AH 272 Mother Population	1.0

Table 3

Resistance to HN_2N-oxide of Clonal Sublines of the Ascites Hepatoma AH 414

No. of Clones	M.E.D. in vivo (mg/kg)
3	10.0
5	5.0
2	2.5
AH 414 Mother Population	5.0

One group of the clones showed the same M.E.D. of the whole cell population of the mother tumor, however, the remaining 2 groups of the clones

manifested their M.E.D. which were higher or lower as compared with that of the mother population. This means that the original population of AH 272 or AH 414 is not a homogeneous but a heterogenous mixture of at least 3 kinds of tumor cells differing from each other in the grade of natural resistance to HN_2N-oxide. The resistance of the original tumor cell population, therefore, is the averaged sum of all that of its constituents which differ from each other in their grade of resistance.

Such a heterogeneity of drug resistance of neoplastic population was also demonstrated by the population analysis of the ascites hepatoma AH 39. As seen from table 4, which showed the M.E.D. in vivo of 11 clonal populations resulted by 95 single-cell transplantations of AH 39, the range of variations of the M.E.D. of these clones was of extraordinary width. The M.E.D. of the mother population of AH 39 was 7.5, whereas the M.E.D. of clones derived from this mother population varied, ranging from 50 to 1. This result also suggests that the original population of AH 39 is of a mosaic constitution of cells of different grades of drug resistance. So it can be noted here that the mother population AH 39 contains these cells showing the high resistance of 50, the same as the maximum tolerance dose of the host.

Table 4

Resistance to HN_2N-oxide of Clonal Sublines of the Ascites Hepatoma AH 39

No. of Clones	M.E.D. in vivo (mg/kg)
1	50.0
1	25.0
2	10.0
4	7.5
2	5.0
1	1.0
AH 39 Mother Population	7.5

Table 5

Resistance to HN_2N-oxide in Clonal Sublines of AH 39 during Serial Animal Passages

Generation / Clones	M.E.D. in vivo (mg/kg)				
	1st	5th	8th	20th	30th
No. 1	50	25	25	5	5
No. 2	25	7.5	5	5	1
No. 3	7.5	7.5	7.5		
No. 4	5	7.5	10	25	5

Table 5 shows the M.E.D. in vivo of 4 different clones of AH 39 during their serial animal passages.

The grade of high resistant clones decreased gradually, while those of lower clones increased by passages through animals. In general, the grade of resistance fluctuated either upwards or downwards around the level of the M.E.D. 7.5 of the original population. This fluctuation was evidently limited within the M.E.D. levels, i.e. 50-1, of the different clones of the mother population. This observation may imply that the natural resistance of a tumor cell population is not a fixed value, but it can fluctuate naturally within a limit corresponding to the range of variations of cells in the population. It may be significant to add the fact that the resistance of original stock population of AH 39 has fluctuated within a relatively narrow range of 10-5 M.E.D. during the prolonged animal passages.

3. Drug resistance of tumor cell population derived from cold-stored tumor cells.

Forty-eight hours' storage of tumor cells in the -80°C. tumor bank was done, using the tumor ascites of AH 39. Then the stored cells were trans-

planted intraperitoneally into normal rats and tumor cell populations that developed were examined for their M.E.D. in vivo. The experiment was repeated 38 times in total. As seen from table 6, the results showed different M.E.D. values which ranged from 50 to 5 that was within the previously mentioned natural range of variation discovered in the established clones.

Table 6

Resistance to HN_2N-oxide of AH 39 After Cold Storage (-80°C. 48hrs.)

No. of Cases	M.E.D. in vivo (mg/kg)
2	50.0
5	25.0
18	10.0
11	7.5
2	5.0
AH 39 (Control)	7.5

The heterogeneity of cancer cell population observed here may not come from the suggestion that different cancer cells possess the definite stable grade of resistance within a neoplastic population but the resistance of the same cells may vary within a natural limit.

V. SUMMARY & CONCLUSION

The natural resistance of tumor cell population against HN_2N-oxide was studied with special respect to heterogeneity of the population employing the ascites hepatoma in the rat. The data so far obtained indicate that;

(1) The real state of drug resistance of tumor cell population is a problem of "grading", when it is considered on the cellular level.

(2) Every tumor has its own peculiar grade of natural drug resistance and the grade is not a fixed value but it can fluctuate naturally around a certain level within a limited range of variations corresponding to the resistance of its cell population. Population analysis of a neoplastic population, after establishing single cell clones from the population, has so far demonstrated that the tumor cell population is not of a homogeneous constitution but is a heterogenous complex of cells of fluctuated grades of drug resistance. The resistance of a tumor cell population, therefore, is the averaged sum of all that of its constituents which differ from each other in their grade of resistance. The drug resistance of tumors can be varied under abnormal conditions such as cold storage at -80°C.

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Figure 1

Figure 2

Figure 3

Explanation of figures.

Figure 1. Phasecontrast microscopic view of the ascites hepatoma AH 272. Individually isolated free tumor cells are abundant, while some clusters of tumor cells are seen.

Figure 2. General view of the asoites hepatoma AH 414. Wright-Giemsa stained smear. Free tumor cells.

Figure 3. Phasecontrast microscopic view of the ascites hepatoma AH 39. Almost all the free tumor cells.